

Septin 4 activates PPAR γ /LXR α signaling by upregulating ABCA1 and ABCG1 expression to inhibit the formation of THP-1 macrophage-derived foam cells

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Abstract. Septin 4 is a member of a family of GTP-binding proteins that has been previously reported regulate cytoskeletal organization. In addition, it has been suggested to serve a role in atherosclerosis. Therefore, the present study aimed to investigate the effects of Septin 4 on foam cell formation. THP-1 cells were first exposed to phorbol-12-myristate-13-acetate for differentiation into macrophages before being transformed into foam cells by treatment with oxidized low-density lipoprotein (ox-LDL). Septin 4 expression was then knocked down or overexpressed in THP-1 cells using transfection, whilst peroxisome proliferator activated receptor γ (PPAR γ) was also inhibited using its selective antagonist (T0070907) in the presence of Septin 4 overexpression. Oil red staining was used to detect lipid uptake, and total cholesterol (TC), free cholesterol (FC) and ATP binding cassette subfamily A/G member 1 (ABCA1/G1) protein expression were also measured. The results demonstrated that upon ox-LDL stimulation, macrophages that were derived from THP-1 cells transformed into foam cells, where Septin 4 was highly expressed in ox-LDL-induced foam cells. Septin 4 knockdown promoted TC and FC levels, but reduced ABCA1/G1 protein expression. The protein expression levels of PPAR γ and liver X receptor α (LXR α) were also decreased after Septin 4 knockdown. However, Septin 4 overexpression resulted in the opposite results being observed. Additionally, blocking PPAR γ activity using its inhibitor T0070907 or knocking down LXR α expression using short hairpin RNA reversed the effects of

Septin 4 overexpression on foam cell formation and cholesterol handling. In conclusion, Septin 4 may serve an important role in preventing foam cell formation by activating PPAR γ /LXR α signaling and subsequently enhancing ABCA1/G1 expression.

Introduction

Atherosclerosis (AS) is one the main pathological causes of cardiovascular disease and is characterized by the accumulation of large numbers of lipids, inflammatory cells and fibrous materials in the vascular endothelium (1). Thrombosis and plaque rupture during AS can result in serious cardiovascular diseases, including coronary heart disease and myocardial infarction (2). Previous epidemiological studies have reported that, in developing countries, especially in China, the prevalence of AS is increasing rapidly (3-5). During the progression of AS, foam cells are one of the main components of the atherosclerotic plaque, where their formation is considered to be the hallmark a key step in AS pathogenesis (6). With the exception of a small subset derived from vascular endothelial cells and smooth muscle cells, the vast majority of foam cells are derived from macrophages that can phagocytose lipids (7). Under physiological conditions, there is a balance between the cholesterol uptake and efflux out of foam cells (7). However, in advanced atherosclerotic plaques, this balance is perturbed and cholesterol efflux decreases (8). This continuous accumulation of free cholesterol (FC) in foam cells can cause inflammatory responses and ultimately lead to foam cell apoptosis (7). Therefore, homeostasis of cholesterol uptake and efflux has a critical importance in AS, where information on the underlying mechanism of foam cell formation and lipid metabolism in macrophages may reveal novel strategies for the treatment of AS.

The peroxisome proliferator activated receptor (PPAR γ /liver X receptor (LXR) α /ATP binding cassette subfamily A member 1 (ABCA1)/ATP binding cassette subfamily G member 1 (ABCG1) pathway in macrophages serves a crucial role in controlling cholesterol efflux (9). Both PPAR γ and LXR α are transcription factors that can be activated by oxidized low-density lipoprotein (ox-LDL), which directly increases the expression levels of the membrane ATP-binding cassette transporters ABCA1 and ABCG1,

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leading to cholesterol excretion from macrophages (10). In addition to regulating cholesterol homeostasis, PPAR γ and LXR α also exhibit anti-inflammatory properties by promoting the release of anti-inflammatory factors, such as TNF α , IL-1 β and IL-6 (11). All these findings aforementioned suggest that activation of PPAR γ and LXR α can alleviate AS. In addition, activation of the PPAR γ /LXR α pathway in foam cells may serve to be a promising anti-AS treatment strategy.

Septin 4 is a member of the septin family that possesses GTPase activity and is widely expressed in eukaryotic cells (12). Septin 4 is considered to be a major component of the cytoskeleton and is involved in numerous important physiological processes, including cell differentiation, vesicle trafficking and apoptosis (12). Septin 4 has also been reported to be a tumor suppressor that can promote the apoptosis of cancer cells. For example, Septin 4 could promote cell death in human colon cancer cells by increasing apoptosis (13). Another study revealed that silencing Septin 4 expression increased platelet-derived growth factor (PDGF)-BB-induced human aortic vascular smooth muscle cell (HAVSMC) proliferation, migration and phenotypic transformation, whilst the overexpression of Septin 4 had the opposite effects, implicating the involvement of Septin 4 in AS (14). However, the role of Septin 4 on foam cell formation, lipid accumulation and lipid metabolism remain poorly understood.

Therefore, the present study intended to investigate the role of Septin 4 in macrophage formation and lipid metabolism and to study any potential underlying mechanism. The expression levels of Septin 4 in ox-LDL-induced foam cells were also measured, following which the effect of Septin 4 knockdown and overexpression on cholesterol accumulation, ABCA1, ABCG and PPAR γ expression was evaluated in foam cells. Mechanistically, PPAR γ and LXR α was knockdown was used to explore the underlying function of Septin 4 on cholesterol accumulation.

Materials and methods

Cell culture and treatment. Human THP-1 monocytes (The Cell Bank of Type Culture Collection of Chinese Academy of Sciences) were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) in a humidified atmosphere with 5% CO₂ at 37°C.

Macrophages were obtained from THP-1 cells stimulated with 100 ng/ml phorbol 12-myristate-13-acetate (PMA; Sigma-Aldrich; Merck KGaA) at 37°C for 48 h. Cells were exposed to ox-LDL (50 μ g/ml) for 24 h.

Lentiviral particles expressing shorthairpinRNA (shRNA/sh) targeting human Septin 4 or LXR α , the corresponding negative control (NC; sh-NC) and the recombinant pcDNA3.0 vector overexpressing Septin 4 (ov-Septin 4) along with the empty pcDNA3.0 vector (empty vector for ov-Septin 4) were designed and successfully constructed by Shanghai GenePharma Co., Ltd. After PMA and ox-LDL treatment, cells were transfected using Lipofectamine[®] 2000 (Thermo Fisher Scientific, Inc.) as described previously (15,16). For PPAR γ inhibition, its selective antagonist T0070907 (10 μ M; Sigma-Aldrich; Merck KGaA) was used to treat transfected or untransfected macrophages at 37°C for 24 h.

Oil red O staining (ORO). THP-1 derived macrophages were exposed to ox-LDL (50 μ g/ml) for 24 h. The cells were washed with a buffer solution (PBS) and directly fixed with 10% formalin for 20 min. Cells were then incubated with 60% isopropanol for 5 min and stained with ORO (Sigma-Aldrich; Merck KGaA) for 30 min. After counterstaining with crystal violet, the cells were imaged using a phase contrast light microscope (magnification, x200; Leica Microsystems GmbH). All operations were performed at room temperature.

Measurement of cholesterol. To measure total cholesterol (TC) and FC levels, THP-1-derived macrophages (1x10⁶) were exposed to ox-LDL (50 μ g/ml) at 37°C for 24 h in six-well plates as aforementioned. TC and FC levels were determined using a TC/FC quantitative method (Abcam; cat. no. ab65359) according to the manufacturer's protocols. Fluorescence measurement was conducted using a microplate reader (Thermo Fisher Scientific, Inc.) at excitation/emission=535/587 nm. TC and FC results are expressed in mg/dl.

Reverse transcription-quantitative qPCR (RT-qPCR). Total RNA were extracted from cells using TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's protocols. Total RNA (~1 μ g) from each sample was then reverse transcribed into cDNA using the PrimeScript[™] RT kit (Takara Bio, Inc.). The temperature protocol for RT was as follows: 37°C for 15 min, followed by 85°C for 5 sec and stop at 4°C. Quantitative assessment of mRNA expression was performed by qPCR based using Fast SYBR[™] Green Master Mix (Thermo Fisher Scientific, Inc.) in an Applied Biosystems (ABI 7500 system; Thermo Fisher Scientific, Inc.). The primers were synthesized by Nanjing Genscript Biotechnology Co., Ltd. The thermocycling conditions were as follows: 95°C for 2 min, followed by 40 cycles of 95°C for 20 sec and 65°C for 40 sec. mRNA expression levels were compared after normalization with β -actin. The primer sequences used were as follows: Septin 4 forward, 5'-CTGCTTTCTTCCTGGATGTCTCT-3' and reverse, 5'-AGGTTCCAAGCCCCAAA-GAAA-3'; LXR α forward, 5'-CCTGGGGATTTGGACAGTGC-3' and reverse, 5'-GCCCTTTTCCGCTTTTGT-3' and β -actin forward, 5'-CTTCTACAATGAGCTGCGTGTG-3' and reverse, 5'-AGT CATAGTCCGCCTAGAAGC-3'. Expression levels of target genes were normalized to the endogenous control GAPDH using the 2^{- $\Delta\Delta$ C_q} method (17)

Western blotting. Total protein was extracted using RIPA lysis buffer (Beyotime Institute of Biotechnology) supplemented with protease and phosphatase inhibitors. After determining the protein concentration using a BCA kit (Thermo Fisher Scientific, Inc.), the proteins (30 μ g per lane) were separated by 10% SDS-PAGE and transferred onto PVDF membranes. The membranes were then blocked with 5% BSA (Beyotime Institute of Biotechnology) solution at room temperature for 1 h and incubated with primary antibodies (All Abcam) against Septin 4 (cat. no. ab166788; 1:2,000), ABCA1 (cat. no. ab66217, 1:1,000), ABCG1 (cat. no. ab52617; 1:5,000), PPAR γ (cat. no. ab178860; 1:1,000), LXR α (cat. no. ab176323; 1:2,000) and GAPDH (ab8245; 1:10,000) overnight at 4°C. After incubation, the membranes were washed, incubated

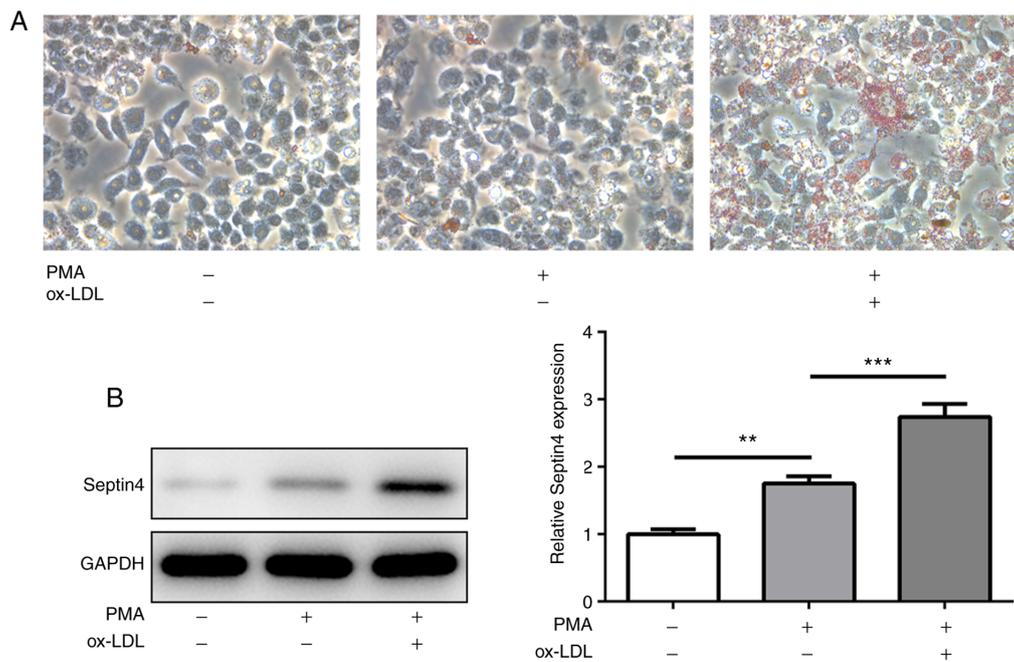


Figure 1. Septin 4 expression is upregulated in THP-1 macrophages upon ox-LDL stimulation. (A) THP-1 cells were treated with PMA to differentiate them into macrophages, followed by incubation with or without 50 $\mu\text{g/ml}$ ox-LDL for 24 h and staining with Oil Red O. Magnification, $\times 200$. (B) Septin 4 protein expression in THP-1 cells treated with or without PMA, followed by incubation with or without 50 $\mu\text{g/ml}$ ox-LDL for 24 h. ** $P < 0.01$ and *** $P < 0.001$. PMA, phorbol-12-myristate-13-acetate; ox-LDL, oxidized low-density lipoprotein.

with the corresponding horseradish peroxidase-conjugated secondary antibodies (goat anti-mouse IgG, cat. no. ab205719, 1:10,000; goat anti-rabbit, cat. no. ab205718, 1:10,000; both Abcam) at room temperature for 2 h and the bands were visualized using an ECL system (Thermo Fisher Scientific, Inc.). Protein expression levels were semi-quantified using Image-Pro Plus software version 6.0 (Media Cybernetic, Inc.).

Statistical analysis. Data are presented as the mean \pm SD from \geq three independent experiments and were analyzed using one-way ANOVA followed by Tukey's test for comparisons. All statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software, Inc.). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Septin 4 expression upregulated in THP-1 macrophages following ox-LDL stimulation. To determine the formation of foam cells derived from THP-1 macrophages, THP-1 monocytes were stimulated with PMA for 48 h to differentiate them into macrophages. Subsequently, the level of intracellular lipid accumulation after ox-LDL treatment was observed using ORO staining. A marked increase in lipid accumulation in PMA-induced THP-1 macrophages was observed upon ox-LDL stimulation compared with cells that were exposed to PMA alone (Fig. 1A), suggesting uptake of lipids in ox-LDL-treated macrophages and the formation of foam cells.

The expression of Septin 4 was subsequently measured. PMA treatment significantly increased Septin 4 expression compared with that in untreated cells, but the additional presence of ox-LDL increased the expression level of Septin 4

further in a significant manner (Fig. 1B). These observations suggest that Septin 4 may serve a role in the formation of foam cells derived from THP-1.

Septin 4 knockdown promotes but Septin 4 overexpression attenuates formation of foam cells derived from THP-1 macrophages. Subsequently, Septin 4 expression was knocked down or overexpressed in THP-1-derived foam cells using the corresponding plasmids. Transfection efficacy was then verified using RT-qPCR. shRNA-Septin 4-1 was chosen to knock down Septin 4 expression due to higher transfection efficacy, which significantly reduced Septin 4 expression compared that in cells transfected with sh-NC (Fig. 2A).

Cellular cholesterol content was then detected, where it was found that Septin 4 knockdown significantly increased intracellular TC and FC concentrations but Septin 4 overexpression resulted in the opposite trend being observed compared with those in their corresponding transfection controls (Fig. 2B and C). To further confirm these results, the uptake of lipids was measured using ORO staining (Fig. 2D). Compared with that in non-transfected ox-LDL-treated THP-1 macrophages, knocking down Septin 4 expression caused a notable increase in the uptake of ox-LDL. By contrast, ORO staining was markedly reduced by the overexpression of Septin 4. These data suggest that Septin 4 can inhibit the formation of foam cells derived from ox-LDL-induced THP-1 macrophages.

Septin 4 knockdown reduces but Septin 4 upregulation enhances the expression levels of ABCA1, ABCG1, PPAR γ and LXR α . The molecular mechanism underlying the actions of Septin 4 was further investigated. The protein expression levels of ABCA1 and ABCG1 were significantly decreased by Septin 4 knockdown in THP-1 macrophages

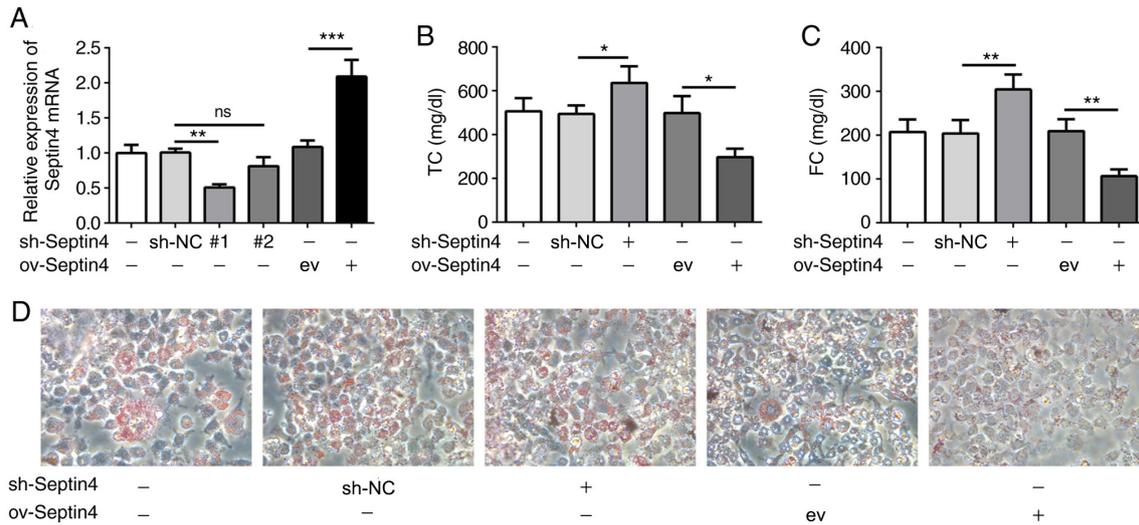


Figure 2. Effect of Septin 4 knockdown and overexpression on cholesterol accumulation in THP-1 macrophage-derived foam cells. (A) Transfection efficiency of Septin 4 knockdown or overexpression in THP-1 cells was assessed using reverse transcription-quantitative PCR. (B) TC and (C) FC content as measured in THP-1 macrophage-derived foam cells following Septin 4 knockdown and overexpression. (D) THP-1 cells transfected with Septin 4 shRNA or overexpression vectors were treated with PMA to differentiate them into macrophages. This was followed by incubation with or without 50 μ g/ml ox-LDL for 24 h and staining with Oil Red O. Magnification, \times 200. * P <0.05, ** P <0.01 and *** P <0.001. TC, total cholesterol; FC, free cholesterol; sh or shRNA, short hairpin RNA; PMA, phorbol-12-myristate-13-acetate; NC, negative control; ov, overexpression; ev, empty vector.

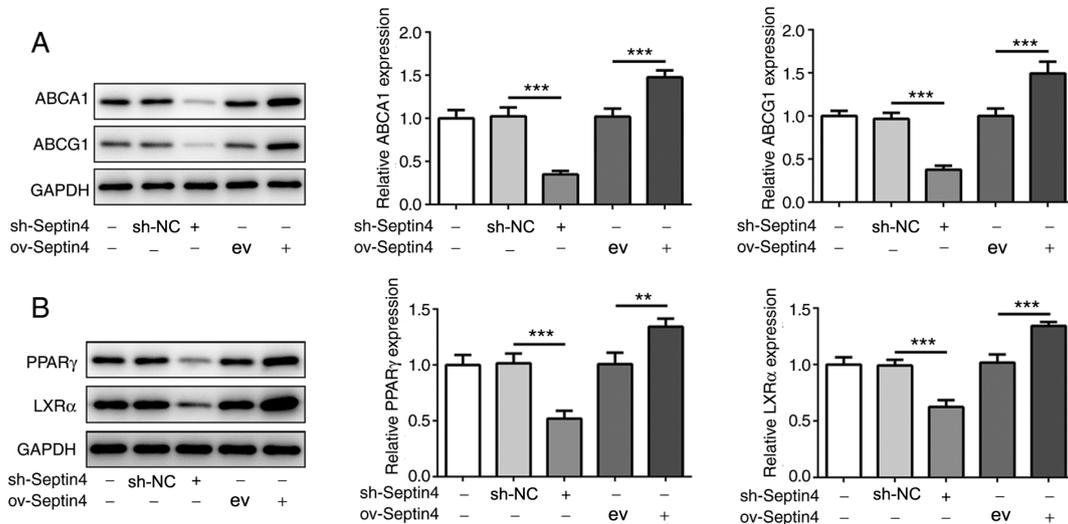


Figure 3. Effect of Septin4 knockdown and overexpression on PPAR γ , LXR α and ABCA1/G1 expression in THP-1 macrophage-derived foam cells. (A) ABCA1, ABCG1, (B) PPAR γ and LXR α protein expression levels in THP-1 macrophage-derived foam cells following Septin 4 knockdown or overexpression were analyzed using western blotting. ** P <0.01 and *** P <0.001. PPAR γ , proliferator activated receptor γ ; LXR α , liver X receptor α ; ABCA1, ATP binding cassette subfamily A member 1; ABCG1, ATP binding cassette subfamily G member 1; NC, negative control; sh, short hairpin; ov, overexpression; ev, empty vector.

compared with those in cells in the sh-NC group (Fig. 3A). By contrast, ABCA1 and ABCG1 protein expression levels were significantly increased by the overexpression of Septin 4 when compared with the empty vector ov-Septin 4 group (Fig. 3A). These results suggest that Septin 4 may inhibit cholesterol accumulation by upregulating ABCA1 and ABCG1 expression, which can assist in excreting cholesterol from the cell.

To further investigate whether PPAR γ and LXR α are involved in the effects of Septin 4, the protein expression levels of PPAR γ and LXR α were also measured. Compared with those in their corresponding transfection controls, Septin 4 knockdown and overexpression significantly reduced and increased the expression levels of PPAR γ and LXR α ,

respectively, compared with those in their corresponding transfection controls (Fig. 3B), consistent with the findings aforementioned. These results suggest that the effects mediated by Septin 4 may involve the PPAR γ /LXR α -related cholesterol metabolism pathway.

Inhibition of PPAR γ or LXR α reverses the effect of Septin 4 overexpression on foam cell formation and ABCA1 and ABCG1 expression. Subsequently, the PPAR γ inhibitor T0070907 and shRNA targeting LXR α were used to inhibit PPAR γ and LXR α , respectively. To avoid off-target effects, two LXR α shRNA candidates were designed and synthesized. Both shRNA-1 and shRNA-2 significantly

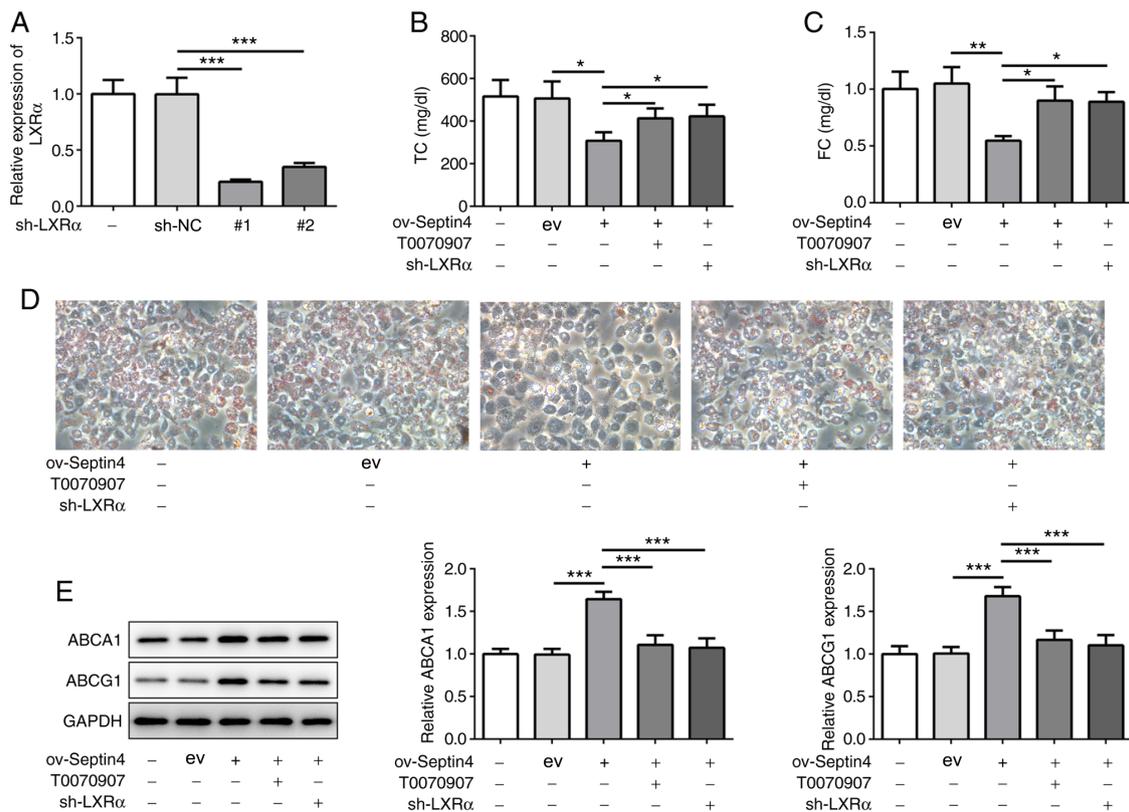


Figure 4. PPAR γ or LXR α inhibition reverses the effect of Septin 4 overexpression on cholesterol accumulation. (A) Transfection efficiency analysis for LXR α knockdown in THP-1 cells was conducted by reverse transcription-quantitative PCR. Measurements of (B) TC and (C) FC content in THP-1 macrophage-derived foam cells following PPAR γ or LXR α inhibition in the presence of Septin 4 overexpression. (D) THP-1 cells following PPAR γ or LXR α inhibition in the presence of Septin 4 overexpression were treated with PMA to differentiate them into macrophages. The macrophages were then incubated with or without 50 μ g/ml ox-LDL for 24 h and stained with Oil Red O. Magnification, x200. (E) ABCA1 and ABCG1 protein expression levels in THP-1 macrophage-derived foam cells following PPAR γ or LXR α inhibition, in the presence of Septin 4 overexpression, were measured by western blotting. *P<0.05, **P<0.01 and ***P<0.001. PPAR γ , proliferator activated receptor γ ; LXR α , liver X receptor α ; ABCA1, ATP binding cassette subfamily A member 1; ABCG1, ATP binding cassette subfamily G member 1; PMA, phorbol-12-myristate-13-acetate; ox-LDL, oxidized low-density lipoprotein; sh, short hairpin; NC, negative control; ov, overexpression; ev, empty vector.

decreased the mRNA expression levels of LXR α compared with those transfected with sh-NC (Fig. 4A). In subsequent experiments, shRNA-LXR α -1 was selected to knockdown LXR α expression in THP-1 macrophages that were overexpressing Septin 4. It was found that the inhibitory effects of Septin 4 overexpression on TC and FC content were significantly reversed by both PPAR γ inhibition or LXR α knockdown (Fig. 4B and C). In addition, similar results were observed in terms of lipid accumulation, where it was found that both PPAR γ inhibition and LXR α knockdown canceled the suppressive effects of Septin 4 overexpression on lipid accumulation (Fig. 4D). PPAR γ inhibition or LXR α knockdown also significantly reversed Septin 4 overexpression-induced increments in ABCA1 and ABCG1 expression (Fig. 4E). Collectively, these findings suggest that the actions of Septin 4 on foam cell formation were dependent on the PPAR γ /LXR α -related cholesterol metabolism pathway.

Discussion

AS is a chronic and maladaptive inflammatory disease that is caused by the accumulation of modified lipoproteins, such as ox-LDL, in the arterial wall (18,19). These lipoproteins can

activate the endothelium, following which activated endothelial cells recruit circulating monocytes to differentiate into macrophages, which then uptake lipoprotein and transform into foam cells to aggravate damage to the blood vessel wall (19). Therefore, preventing the accumulation of cholesterol in macrophages in addition to the transformation of macrophages into foam cells may be beneficial in alleviating AS.

The present study demonstrated that the expression of Septin 4 was upregulated in PMA-stimulated THP-1 macrophages, which was increased further upon ox-LDL stimulation, suggesting that Septin 4 expression was gradually enhanced during the formation of THP-1-derived foam cells. This result was consistent with another recent finding, which revealed that Septin 4 was highly expressed in ApoE $^{-/-}$ AS mice and PDGF-BB-induced HAVSMCs, where it could prevent PDGF-BB-induced HAVSMC phenotypic transformation, proliferation and migration (14). In addition, Wang *et al* (20) previously reported that Septin 4 expression was upregulated in the mouse aorta and cultured vascular smooth muscle cells (VSMCs) following angiotensin-II stimulation, thus regulating Angiotensin-II mediated VSMCs proliferation and migration. The proliferation and migration of VSMCs are the core processes leading to AS (21). In response to certain stimuli, VSMCs exhibit aberrant proliferation, migration and extracellular

matrix (ECM) synthesis, which will promote plaque formation and contribute to the progression of hypertension, AS and other vascular diseases (22). Therefore, in accordance with previous reports, the present findings provided evidence that Septin 4 served an inhibitory role in the development of AS, such that upregulation of Septin 4 may protect cells against cholesterol-induced injury. The present study also knocked down and overexpressed Septin 4 in THP-1 macrophages in the presence of ox-LDL treatment. The results demonstrated that knocking down Septin 4 expression can promote cholesterol accumulation in THP-1 macrophages and then formation of foam cells. By contrast, Septin 4 overexpression resulted in the opposite effects being observed. Therefore, it can be suggested that Septin 4 served as an inhibitor of the formation of foam cells from THP-1 macrophages.

In the present study, Septin 4 overexpression elevated, whilst its knockdown reduced ABCA1 and ABCG1 expression. Previous studies reported that ABCA1/G1 serves a key role in promoting cellular cholesterol efflux and regulating lipid metabolism (9,23). In total, >50% cholesterol is excreted from macrophages by ABCA1 and ABCG1, whereby in advanced plaques, reduced expression levels of ABCA1 and ABCG1 typically leads to an 80% reduction in cholesterol efflux and increases in lipid accumulation (8). The expression of ABCA1/G1 can be regulated by a large network of transcription factors, including PPAR γ and LXR α , which can be activated by ox-LDL and directly increases the expression of ABCA1/G1 (24). It has been previously reported that PPAR γ and LXR α agonists can promote cholesterol efflux from macrophages and relieve AS (25). In addition, the present study suggests that overexpression of Septin 4 could upregulate PPAR γ and LXR α expression levels but Septin 4 knockdown exerted opposite effects. These findings support notion that Septin 4 may enhance ABCA1/G1 expression by activating PPAR γ /LXR α signaling.

To further verify the present hypothesis, T0070907 was used to inhibit PPAR γ and shRNA was utilized to silence LXR α expression in ox-LDL treated THP-1 macrophages in the presence of Septin 4 overexpression. The inhibitory effects of Septin 4 overexpression on cholesterol accumulation and its positive effects on ABCA1/G1 expression were reversed by both PPAR γ and LXR α inhibition. This finding supports the notion of an important role of PPAR γ /LXR α signaling in mediating the actions of Septin 4 on cholesterol accumulation in THP-1 macrophages. However, the present study only displayed *in vitro* findings. *In vivo* animal models or human samples will need to be applied in future studies to validate the conclusions in the present study.

In conclusion, the present study suggests that Septin 4 was involved in preventing THP-1 macrophage transformation into foam cells stimulated by ox-LDL through the activation of PPAR γ /LXR α signaling, thereby increasing ABCA1/G1 expression. The present study provided a potentially novel regulation pathway of ox-LDL-induced foam cell formation and demonstrated that Septin 4 upregulation may be a new target for preventing foam cell formation during the development of AS.

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Availability of data and materials

All datasets generated and/or analyzed during the present study are included in this published article.

Authors' contributions

XYS and GLY conceived, designed the study and acquired the data. HHW analyzed the data. DFL drafted the manuscript and revised it for critically important intellectual content. All authors read and approved the final manuscript. YYS and GLY confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interest

The authors declare that they have no competing interests.

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